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Novel direct and indirect cyclin-dependent kinase modulators for the prevention and treatment of human neoplasms

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Abstract Abnormalities in the cell cycle are responsible for the majority of human neoplasias. Most abnormalities occur due to hyperphosphorylation of the tumor suppressor gene Rb by the key regulators of the cell cycle, the cyclin-dependent kinases (CDKs). Thus, a pharmacological CDK inhibitor may be useful in the prevention and/or treatment of human neoplasms. Flavopiridol is a flavonoid with interesting preclinical properties: (1) potent CDK inhibitory activity; (2) it depletes cyclin D1 and vascular endothelial growth factor mRNA by transcriptional and posttranscriptional mechanisms, respectively; (3) it inhibits positive elongation factor B, leading to transcription "halt"; and (4) it induces apoptosis in several preclinical models. The first phase I trial of a CDK inhibitor, flavopiridol, has been completed. Dose-limiting toxicities included secretory diarrhea and proinflammatory syndrome. Antitumor activity was observed in some patients with non-Hodgkin's lymphoma and renal, colon, and prostate cancers. Concentrations between 300 and 500 n*M*—necessary to inhibit CDK—were achieved safely. Phase II trials with infusional flavopiridol and phase I infusional trials in combination with standard chemotherapy are being completed with encouraging results. A novel phase I trial of 1-h flavopiridol administration was recently completed. The maximum tolerated doses using flavopiridol daily for 5, 3, and 1 consecutive days are 37.5, 50, and 62.5 mg/m^2 per day. Dose-limiting toxicities include vomiting, neutropenia, proinflammatory syndrome, and diarrhea. Plasma flavopiridol concentrations achieved were in the range 1.5–3.5 μM. Phase II/III trials using this 1-h schedule in several tumor types including non-small-cell lung cancer, chronic lymphocytic leukemia, mantle cell lymphoma, and head and neck cancer are being conducted worldwide. UCN-01, the second CDK modulator that has entered clinical trials, has unique preclinical properties: (1) it inhibits protein kinase C (PKC) activity; (2) it promotes cell-cycle arrest by accumulation in p21/p27; (3) it induces apoptosis in several preclinical models; and (4) it abrogates the G_2 checkpoint by inhibition of chk1. The last of these represents a novel strategy to combine UCN-01 with DNA-damaging agents. In the initial UCN-01 clinical trial (continuous infusion for 72 h), a prolonged half-life of about 600 h (100 times longer than in preclinical models) was observed. The maximum tolerated dose was 42.5 mg/m^2 per day for 3 days. Dose-limiting toxicities were nausea/vomiting, hypoxemia, and symptomatic hyperglycemia. One patient with melanoma achieved a partial response (8 months). Another patient with refractory anaplastic large-cell lymphoma had no evidence of disease at >4 years. Bone marrow and tumor samples obtained from some patients revealed loss in adducin phosphorylation, a substrate of PKC. Phase I trials with shorter infusions are being completed. In summary, the first two CDK modulators have shown encouraging results in early clinical trials. A question that remains unanswered is "Which is the best schedule for combination with standard antitumor agents?" Moreover, it is still unclear which pharmacodynamic endpoint reflects loss of CDK activity in tissue samples from patients in these trials. Despite these caveats, we feel that CDKs are sensible targets for cancer therapy and that there are several small-molecule CDK modulators in clinical trials with encouraging results.

Keywords Cell cycle · Flavopiridol · Cyclin-dependent kinases · Clinical trials · Apoptosis

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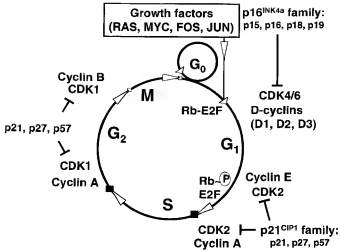

Fig. 1 Cell-cycle regulation; the four phases of cell-cycle progression (*CDK* cyclin-dependent kinases, *Rb* retinoblastoma protein)

Cell-cycle regulation and the role of the cell cycle in carcinogenesis

On activation of several growth factor/mitogenic signaling cascades, cells commit to entry into a series of regulated steps allowing traverse of the cell cycle. First, synthesis of DNA (genome duplication), also known as S phase, occurs followed by separation of two daughter cells (chromatid separation) or M phase. The time between the S and M phases is known as the G_2 phase (Fig. 1). This period is when cells can repair errors that occur during DNA duplication, preventing the propagation of these errors to daughter cells. In contrast, the G_1 phase represents the period of commitment to cell-cycle progression that separates M and S phases as cells prepare for DNA duplication on mitogenic signals [101, 136].

Regulation of the cell cycle and proliferation has been extensively studied in the last few years and a consensus paradigm of cell-cycle regulation has been developed [101, 136]. According to this paradigm, the master switch of the cell cycle is the retinoblastoma (Rb) family of proteins. Proliferation occurs when Rb is phosphorylated and inactivated by serine/threonine kinases known as cyclin-dependent kinases (CDKs) (Fig. 1) [136]. These kinases are activated by D-type cyclins (D1, D2, and D3) and cyclin E, and inhibited by two families of CDK inhibitors, the INK ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, $p19^{INK4d}$) and CIP/KIP families ($p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$) [137].

Rb proteins are pocket proteins that sequester E2F transcription factors, preventing them from activating critical genes in cell proliferation. In addition, Rb/E2F binds to histone deacetylase to form complexes that act as transcriptional repressors [154, 166]. After Rb phosphorylation by CDK4 and/or CDK6 complexes during G_1 phase and CDK2 at G_1/S interphase, E2F proteins are released and promote the transcription of genes essential for the transition to S phase of the cell cycle [38, 80]. CDK4,6/D-type cyclins therefore execute their critical functions during mid-to-late G_1 phase, as cells cross a G_1 restriction point and become independent of mitogens for completion of the division cycle. These features suggest that the fundamental role of these complexes is to integrate extracellular signals with the cell-cycle machinery [101, 136]. CDKs clearly involved in cell-cycle control are CDK1 through 7. In contrast, CDK8 and CDK9, although structurally related to the cell-cycle regulatory CDKs, are important regulators of transcriptional control [104, 159]. There are at least 15 different known cyclins (cyclin A through T) [44, 55, 83, 100]. Cyclin expression varies during the cell cycle and indeed their periodic expression forms the basis for defining the start and transition to succeeding cell-cycle phases. When cyclins noncovalently form 1:1 complexes with their cognate CDK catalytic subunits to form the CDK holoenzyme, the complex becomes activated by phosphorylation in specific residues of the catalytic subunit of the CDK by CDK7/cyclin H, also known as CDK-activating kinase (CAK) [68, 148].

Other important points of regulation have been described in G_2 and mitosis. In these phases also, the specific expression of certain regulators is essential to control the correct sequence of events that lead to cell division. Basically, the cyclins B1, B2 and its partner CDC2 (CDK1), together with other kinases and phosphatases (WEE1, CDC25) regulate the final phases of the cell cycle (Fig. 1). For further insight into cell-cycle regulation, excellent reviews of cell-cycle control have been published recently [39, 54, 92, 102].

Most human neoplasms have abnormalities in some component of the Rb pathway due either to hyperactivation of CDKs as a result of amplification/overexpression of positive cofactors, cyclins/CDKs, or to downregulation of negative factors, endogenous CDK inhibitors or mutation in the Rb gene product. These aberrations promote deregulated S-phase progression in a way that ignores growth factor signals, with loss of G_1 checkpoints [136, 160]. Therefore development of pharmacological small-molecule CDK inhibitors (smCDKI), "mechanism-based therapy," would be of great interest as a treatment strategy for many neoplasms [121, 123, 125]. Furthermore, inappropriate or deregulated activation of CDKs might have adverse consequences for cells, and indeed CDK activation/inactivation has been reported to correlate with cellular response to apoptotic stimuli in several preclinical models [28, 69, 88, 139]. Two CDK modulators, flavopiridol and UCN-01, have completed initial human phase I trials [37, 111, 116, 125, 126, 128, 130, 146, 147, 150] and are described below.

Perturbation of cell-cycle component in neoplastic diseases

In the past three decades, it has become apparent that neoplastic cells display alterations in the progression of

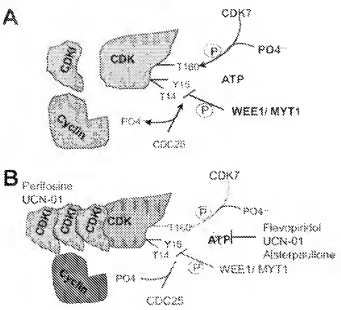

Fig. 2A, B Modes of action for cell-cycle modulators: direct effects on the catalytic CDK subunit (**A**) or indirect modulation of regulatory pathways that govern CDK activity (**B**). Loss in CDK function may occur due to loss in mass of catalytic subunit and/or cofactors, increased endogenous inhibitors, by increased WEE1/MYT1, or by loss in CDK7 or CDC25c activity. However, the most successful way to modulate CDK is by competing with ATP binding in CDK (*CDK* cyclin-dependent kinase, *CDKI* CDK inhibitor)

the normal cell cycle [33, 60, 61, 93, 136]. Cancer cells become malignant as a consequence of activating (i.e. gain-of-function) mutations and/or increased expression of one or more cellular protooncogenes, and/or inactivating (i.e. loss-of-function) mutations and/or decreased expression of one or more tumor suppressor genes. Most tumor suppressor genes and oncogenes are components of signal transduction pathways that control crucial cellular functions, including cell-cycle entry/exit. In contrast to normal cells, tumor cells are unable to stop at predetermined points of the cell cycle, so-called "checkpoints." These pauses in the cell cycle are necessary to verify the integrity of the genome before cells advance to the next phase [46, 99]. Interestingly, critical activities of tumor suppressor genes ultimately regulate these checkpoints.

Therapeutic approaches for the manipulation of the cell-cycle machinery

Several strategies could be considered to modulate CDK activity (Fig. 2). These strategies are divided into direct effects on the catalytic CDK subunit or indirect modulation of regulatory pathways that govern CDK activity [120, 125]. As depicted in Fig. 2A, the smCDKIs are compounds that directly target the catalytic CDK subunit. Most of these compounds modulate CDK activity by interacting specifically with the ATP-binding site of CDKs [36, 86, 120, 125, 168]. Examples of this class include flavopiridol, UCN-01, and alsterpullone (see Tables 1 and 2). The second class of CDK inhibitors are compounds that inhibit CDK activity by targeting the regulatory upstream pathways that modulate CDK activity by: altering the expression and synthesis of the CDK/cyclin subunits or the CDK inhibitory proteins; modulating the phosphorylation of CDKs; targeting *CAK*, *CDC25*, and *WEE1/MYT1*; or manipulating the proteolytic machinery that regulates the catabolism of CDK/cyclin complexes or their regulators (Fig. 2B) [120, 125]. Examples of this class of compounds include perifosine and UCN-01, among others (see Tables 1 and 2).

Modulators of CDK activity

As mentioned above, CDKs can be modulated by direct effects on the catalytic subunit and/or by disruption of upstream regulatory pathways. Several examples and mechanisms are listed in Tables 1 and 2 as well as described in the literature [85, 120, 121, 122, 124].

Modulators of CDKs in clinical trials

Flavopiridol

Mechanism of antiproliferative effects

Flavopiridol (L86-8275 or HMR 1275) is a semisynthetic flavonoid derived from rohitukine, a plant indigenous to India. Initial studies with flavopiridol demonstrated modest in vitro inhibitory activity with respect to epidermal growth factor receptor (EGFR) and protein kinase A ($IC_{50} = 21$ and 122 μM, respectively) [118]. However, when this compound was tested in the US National Cancer Institute (NCI) 60 cell-line anticancer drug screen, it demonstrated a potent growth inhibition ($IC_{50} = 66$ nM), a concentration that is about 1000 times lower than the concentration required to inhibit protein kinase A and EGFR [118]. Initial studies with this flavonoid revealed clear evidence of G_1/S or G_2/M arrest due to loss in CDK1 and CDK2 [71, 81, 164]. Studies using purified CDKs showed that the inhibition observed is reversible and competitively blocked by ATP with a Ki of 41 nM [22, 23, 71, 81, 164]. Furthermore, the crystal structure of the complex of deschloroflavopiridol and CDK2 showed that flavopiridol binds to the ATP-binding pocket, with the benzopyran occupying the same region as the purine ring of ATP [35], confirming earlier biochemical studies with flavopiridol [81]. Flavopiridol inhibits all CDKs thus far examined (IC_{50} about 100 nM) but inhibits CDK7 (CAK) less potently (IC_{50} about 300 nM) [22, 23, 81].

In addition to directly inhibiting CDKs, flavopiridol promotes a decrease in the level of cyclin D1, an oncogene that is overexpressed in many human neoplasias.

Table 1 Indirect CDK modulators

Mechanism for loss in CDK activity	Examples	References
ATP-binding pocket competition	Direct CDK inhibitors (see Table 2)	
Overexpression of endogenous CDK inhibitors		
Gene therapy	$p16^{INK4a}$	30, 66
	$p53/p21^{CIP1}$	43, 45, 107, 140
	$p27^{KIP1}$	34, 70
	Lovastatin	59
	Rapamycin	62
	UCN-01	5, 98
	Perifosine	97
Small molecules	p16-derived peptides	47, 53
	p21-derived peptides	13, 158
Peptidomimetic-based	Peptides that block cyclin/CDK contact	27, 32
Depletion of CDK/cyclins		
Antisense approaches	Cyclin D1	21, 42, 77, 155
	Tamoxifen	169
	Rapamycin	62, 91
	Lovastatin	31, 59
Small molecules	Retinoids	162
	Flavopiridol	24
Modulation of proteasomal machinery	PS341	1
Modulation of upstream phosphatases/kinases	Caffeine	48
	Fostriecin	105
	Dysidiolide	12
	Others	8, 40

CDK cyclin-dependent kinase

Table 2 Direct CDK modulators

Specificity	Examples	References
CDK1/CDK2/CDK5	Roscovitine	58, 87
	Olomucine	18, 58, 114
	CVT-313	14
	Butyrolactone I	75
	Purvalanol and compound 52	57, 106
Nonspecific CDK	Flavopiridol	119, 125
	Staurosporine	2, 119
	UCN-01	2, 4, 125, 131, 132
	Oxyndole I	73
Unknown	Toyocamycin	94
	Paullones	58, 113, 168
	Myricetin	153

CDK cyclin-dependent kinase

Neoplasms that overexpress cyclin D1 have a poor prognosis [50, 52, 90]. When MCF-7 human breast carcinoma cells were incubated with flavopiridol, levels of cyclin D1 protein decreased within 3 h [24]. This effect was followed by a decline in the levels of cyclin D3 with no alteration in the levels of cyclin D2 and cyclin E, the remaining G_1 cyclins, leading to loss in the activity of CDK4. Thus depletion of cyclin D1 appears to lead to the loss of CDK activity [24]. The depletion of cyclin D1 is caused by depletion of cyclin D1 mRNA and was associated with a specific decline in cyclin D1 promoter measured by a luciferase reporter assay [24]. The transcriptional repression of cyclin D1 observed after treatment with flavopiridol is consistent with the effects of flavopiridol on yeast cells, and underscores the conserved effect of flavopiridol on eukaryotic cyclin transcription [57].

In summary, flavopiridol can induce cell-cycle arrest by at least three mechanisms: (1) direct inhibition of CDK activities by binding to the ATP-binding site; (2) prevention of the phosphorylation of CDKs at Thr160/161 by inhibition of CDK7/cyclin H [22, 164]; and (3) decrease in the amount of cyclin D1, an important cofactor for CDK4 and CDK6 activation (G_1/S arrest only). Another effect of flavopiridol on transcription is attenuation of the induction of vascular endothelial growth factor (VEGF) mRNA in monocytes after hypoxia (the antiangiogenic properties of flavopiridol are described below). This effect is due to alterations in the stability of VEGF mRNA [89].

Chao et al. have demonstrated that flavopiridol potently inhibits positive elongation factor B (P-TEFb; also known as CDK9/cyclin T) with a Ki of 3 n*M*, leading to inhibition of transcription by blocking the transition into productive elongation [25]. Interestingly, in contrast with all CDKs tested so far, flavopiridol appears non-competitive with ATP in this reaction. P-TEFb is a required cellular cofactor for the human immunodeficiency virus 1 (HIV-1) transactivator, Tat. Consistent with its ability to inhibit P-TEFb, flavopiridol blocked Tat transactivation of the viral promoter in vitro. Furthermore, flavopiridol blocked HIV-1 replication in both single-round and viral-spread assays with an IC_{50} of < 10 n*M* [25]. These actions of the drug led to the testing of flavopiridol through clinical trials for patients with HIV-related malignancies [165].

An important biochemical effect involved in the antiproliferative effect of flavopiridol is the induction of

apoptotic cell death. Hematopoietic cell lines are often sensitive to flavopiridol-induced apoptotic cell death [7, 20, 76, 95], but the mechanism(s) by which flavopiridol induces apoptosis have not yet been elucidated. Flavopiridol does not modulate topoisomerase I/II activity [95]. In certain hematopoietic cell lines, neither BCL-2/BAX nor p53 appeared to be affected [95, 135], whereas in other systems BCL-2 may be inhibited [76]. Preliminary evidence from one laboratory demonstrated that flavopiridol-induced apoptosis in leukemia cells is associated with early activation of the MAPK protein kinase family of proteins (MEK, p38, and JNK) [78]. This activation may lead to activation of caspases [78]. As seen in this and other models, caspase inhibitors prevent flavopiridol-induced apoptosis [20, 78]. It is unclear whether the putative flavopiridol-induced inhibition of CDK activity is required for induction of apoptosis.

Clear evidence of cell-cycle arrest along with apoptosis was observed in a panel of squamous head and neck cancer cell lines, including a cell line (HN30) that is refractory to several DNA-damaging agents such as γ-irradiation and bleomycin [96]. Again, the apoptotic effect was independent of p53 status and was associated with the depletion of cyclin D1 [96]. These findings have been corroborated in other preclinical models [10, 29, 112, 135]. Efforts to understand flavopiridol-induced apoptosis are under intense investigation.

Flavopiridol targets not only tumor cells but also angiogenesis pathways. Brusselbach et al. [15] incubated primary human umbilical vein endothelial cells with flavopiridol and observed apoptotic cell death even in cells that were not cycling, leading to the notion that flavopiridol may have antiangiogenic properties due to endothelial cytotoxicity. In other model systems, Kerr et al. [74] tested flavopiridol in an in vivo Matrigel model of angiogenesis and found that flavopiridol decreased blood vessel formation, a surrogate marker for antiangiogenic effect of this compound. Furthermore, as mentioned above, Melillo et al. [89] demonstrated that, at low nanomolar concentrations, flavopiridol prevented the induction of VEGF by hypoxic conditions in human monocytes. This effect was caused by a decreased stability of VEGF mRNA, which paralleled the decline in VEGF protein. Thus the antitumor activity of flavopiridol observed may be in part due to antiangiogenic effects. Whether the various antiangiogenic actions of flavopiridol result from its interaction with a CDK target or other targets requires further study.

The antitumor effect observed with flavopiridol can also be explained by activation of differentiation pathways. It became clear recently that cells become differentiated when exit from the cell cycle (G_0) and loss of CDK2 activity occurs. Based on this information, Lee et al. [79] tested flavopiridol and roscovitine, both known CDK2 inhibitors, to determine if they induce a differentiated phenotype. For this purpose, NCI-H358 lung carcinoma cell lines were exposed to CDK2 antisense construct, flavopiridol, or roscovitine. Clear evidence of mucinous differentiation along with loss in CDK2 activity was observed. However, each CDK2-antagonist therapy had different cell-cycle regulatory expression despite a similar differentiated phenotype [79].

Several investigators have attempted to determine whether flavopiridol has synergistic effects with standard chemotherapeutic agents. For example, synergistic effects in A549 lung carcinoma cells were demonstrated when treatment with flavopiridol followed treatment with paclitaxel, cytarabine, topotecan, doxorubicin, or etoposide [11, 115]. In contrast, a synergistic effect was observed with 5-fluorouracil only when cells were treated with flavopiridol for 24 h before addition of 5-fluorouracil. Furthermore, synergistic effects with cisplatin were not schedule-dependent [11]. However, Chien et al. [29] failed to demonstrate a synergistic effect between flavopiridol and cisplatin and/or γ-irradiation in bladder carcinoma models. One important issue is that most of these combination studies were performed in in vitro models. Thus confirmatory studies in in vivo animal models are needed.

Experiments using colorectal (Colo205) and prostate (LnCap or DU-145) carcinoma xenograft models in which flavopiridol was administered frequently over a protracted period demonstrated that flavopiridol is cytostatic [41, 118]. These demonstrations led to human clinical trials of flavopiridol administered as a 72-h continuous infusion every 2 weeks [127] (see below). Subsequent studies in human leukemia/lymphoma xenografts demonstrated that flavopiridol administered intravenously as a bolus rendered animals tumor-free, whereas flavopiridol administered as an infusion only delayed tumor growth [7]. Moreover, in head and neck (HN-12) cancer xenografts, flavopiridol administered as an intraperitoneal bolus daily at 5 mg/kg for 5 days demonstrated a substantial growth delay [96]. Again, apoptotic cell death and cyclin D1 depletion were observed in tissues from xenografts treated with flavopiridol [7]. Based on these results, a phase I trial of 1-h daily infusional flavopiridol every 3 weeks was conducted at the NCI [147].

Clinical experience with flavopiridol

Two phase I clinical trials of flavopiridol administered as a 72-h continuous infusion every 2 weeks have been completed [127, 150]. In the NCI phase I trial ($n = 76$) of infusional flavopiridol, dose-limiting toxicity was secretory diarrhea with a maximum tolerated dose of 50 mg/m^2 per day for 3 days. In the presence of antidiarrheal prophylaxis (a combination of cholestyramine and loperamide), patients tolerated higher doses, defining a second maximum tolerated dose of 78 mg/m^2 per day for 3 days. The dose-limiting toxicity observed at the higher MTD level was a substantial proinflammatory syndrome (fever, fatigue, local tumor pain, and modulation of acute-phase reactants) and reversible hypo-

tension [127]. Minor responses were observed in patients with non-Hodgkin's lymphoma, and colon or kidney cancer for >6 months. Moreover, one patient with refractory renal cancer achieved a partial response for >8 months [127]. Of 14 patients who received flavopiridol for >6 months, five received flavopiridol for >1 year and one received flavopiridol for >2 years [127]. Plasma concentrations of 300–500 n*M* flavopiridol, which inhibit CDK activity in vitro, were safely achieved during this trial [127].

In a complementary phase I trial also exploring the same schedule (72-h continuous infusion every 2 weeks), Thomas et al. [150] found that the dose-limiting toxicity was diarrhea, corroborating the NCI experience. Moreover, plasma concentrations of 300–500 n*M* flavopiridol were also observed. Interestingly, there was one patient in this trial with refractory metastatic gastric cancer who had progressed after a treatment regimen containing 5-fluorouracil. When treated with flavopiridol, this patient achieved a sustained complete response without any evidence of disease for >2 years after treatment was completed.

The first phase I trial of a daily 1-h infusion of flavopiridol for five consecutive days every 3 weeks has been completed [147]. This schedule was based on antitumor results observed in leukemia/lymphoma and head and neck cancer xenografts treated with flavopiridol [7, 96]. A total of 55 patients were treated in this trial. The recommended phase II dose is 37.5 mg/m^2/day for five consecutive days. Dose-limiting toxicities observed at 52.5 mg/m^2 per day are nausea/vomiting, neutropenia, fatigue, and diarrhea [147]. Other (non-dose-limiting) adverse effects are local tumor pain and anorexia. To reach higher flavopiridol concentrations, the protocol was amended to administer flavopiridol for 3 days and then for 1 day only. With these protocol modifications we were able to achieve concentrations (about 4 μM) necessary to induce apoptosis in xenograft models [7, 96, 147]. The half-life observed in this trial was much shorter (about 3 h) than the infusional trial (about 10 h). Thus the high micromolar concentrations achieved in the 1-h infusional trial could be maintained for short periods [147]. Several phase II trials in patients with refractory head and neck cancer, chronic lymphocytic leukemia (CLL), and mantle cell lymphoma (MCL) are underway using this schedule (see below). A phase I trial testing the combination of paclitaxel and infusional (24-h) flavopiridol demonstrated good tolerability with a dose-limiting pulmonary toxicity [117].

Phase II trials of flavopiridol given as a 72-h continuous infusion with the maximum tolerated dose in the absence of antidiarrheal prophylaxis (50 mg/m^2/day) to patients with CLL, non-small-cell lung cancer, non-Hodgkin's lymphoma, and colon, prostate, gastric, head and neck, and kidney cancer, and phase I trials of flavopiridol administered on novel schedules and in combination with standard chemotherapeutic agents are being performed [9, 134, 141, 161, 165]. In a phase II trial of flavopiridol in metastatic renal cancer, two objective responses (response rate = 6%, 95% CI 1–20%) were observed. Most patients developed grade 1/2 diarrhea and asthenia [141]. In this trial, patients who demonstrated glucuronide flavopiridol metabolites in plasma, as measured by high-performance liquid chromatography methodology, have less pronounced diarrhea in comparison to nonmetabolizers [65]. Thus it may be possible that patients with higher metabolic rates may tolerate higher doses of flavopiridol.

Phase II trials of shorter (1-h) infusional flavopiridol are being conducted in MCL, CLL, and head and neck squamous cell carcinoma. Several patients with refractory CLL and MCL demonstrated clear evidence of responses (partial responses) in these trials (Dr. Jose Ramon Suarez, Aventis Corporation, personal communication).

Although the initial studies of flavopiridol in humans are encouraging, the best schedule of administration of flavopiridol needs to be determined. Furthermore, phase III studies in combination with standard chemotherapy are being considered (Jose Ramon Suarez, personal communication).

UCN-01

Mechanism of antiproliferative activity

Staurosporine is a potent nonspecific protein and tyrosine kinase inhibitor with a low therapeutic index in animals [145]. Thus efforts to find analogs of staurosporine have identified compounds specific for protein kinases. One staurosporine analog, UCN-01 (7-hydroxystaurosporine), has potent activity against several protein kinase C (PKC) isoenzymes, particularly the Ca^{2+}-dependent PKC with an IC_{50} of about 30 n*M* [132, 143, 144]. In addition to its effects on PKC, UCN-01 has antiproliferative activity in several human tumor cell lines [2, 4, 5, 131, 156]. In contrast, another highly selective potent PKC inhibitor, GF 109203X, has minimal antiproliferative activity, despite a similar capacity to inhibit PKC in vitro [156]. These results suggest that the antiproliferative activity of UCN-01 cannot be explained solely by inhibition of PKC. Although UCN-01 moderately inhibited the activity of immunoprecipitated CDK1 (CDC2) and CDK2 (IC_{50} = 300–600 n*M*), exposure of intact cells to UCN-01 leads to "inappropriate activation" of the same kinases [156]. This phenomenon correlates with the G_2 abrogation checkpoint observed with this agent.

Experimental evidence suggests that DNA damage leads to cell-cycle arrest to allow DNA repair. In cells where the G_1 phase checkpoint is not active because of p53 inactivation, irradiated cells accumulate in G_2 phase due to activation of the G_2 checkpoint (inhibition of CDC2). In contrast, Wang et al. exposed CA46 cell lines to radiation followed by UCN-01, promoting the inappropriate activation of CDC2/cyclin B and early mitosis with the onset of apoptotic cell death [157]. These effects

could be partially explained by the inactivation of WEE1, the kinase that negatively regulates the G_2/M-phase transition [167]. Moreover, UCN-01 can have a direct effect on chk1, the protein kinase that regulates the G_2 checkpoint [19, 56, 108]. Thus although UCN-01 at high concentrations can directly inhibit CDKs in vitro, UCN-01 can modulate cellular "upstream" regulators at much lower concentrations, leading to inappropriate CDC2 activation. Studies from other groups suggest that not only is UCN-01 able to abrogate the G_2 checkpoint induced by DNA-damaging agents but also, in some circumstances, UCN-01 is able to abrogate the DNA damage-induced S-phase checkpoint [17, 133].

Another interesting property of UCN-01 is its ability to arrest cells in G_1 phase of the cell cycle [4, 5, 6, 26, 72, 131, 138, 152]. When human epidermoid carcinoma A431 cells (mutated p53) or HN12 head and neck carcinoma cell lines were incubated with UCN-01, these cells were arrested in G_1 phase with Rb hypophosphorylation and $p21^{WAF1}/p27^{KIP1}$ accumulation [5, 98]. Chen et al. suggest that Rb, but not p53, function is essential for UCN-01-mediated G_1 arrest [26]. However, Shimizu et al. demonstrated that lung carcinoma cell lines with either absent, mutant, or wild-type Rb exposed to UCN-01 displayed G_1 arrest and antiproliferative effects irrespective of Rb function [138]. Thus the exact role of Rb or p53 in the G_1 arrest induced by UCN-01 remains unknown. Further studies on the putative target(s) for UCN-01 in the G_1 phase arrest of cells are warranted.

Another interesting pharmacological feature of UCN-01 is the observed increased cytotoxicity in cells that harbor mutated p53 [157]. In CA-46 and HT-29 tumor cell lines carrying mutated p53 genes, exposure to UCN-01 results in potent cytotoxicity. To extend these observations further, the MCF-7 cell line with no endogenous p53 because of the ectopic expression of E6, a human papillomavirus type-16 protein, showed enhanced cytotoxicity when treated with a DNA-damaging agent, such as cisplatin, and UCN-01, compared with the isogenic wild-type MCF-7 cell line [157]. Thus a common feature observed in >50% of human neoplasias associated with poor outcome and refractoriness to standard chemotherapies [82, 84] may render tumor cells more sensitive to UCN-01.

An exciting development is the reported effects of UCN-01 on the PI3 kinase/AKT survival pathway [109, 149]. UCN-01 displays a potent inhibition in vitro of the phoshoinositide-dependent kinase 1 serine/threonine kinase, leading to dephosphorylation and inactivation of AKT [109]. Although this is an exciting novel feature of UCN-01, it is of utmost importance to demonstrate whether the antitumor effects of UCN-01 are mediated by this action. Moreover, demonstration that this effect also occurs in in vivo settings is crucial.

As mentioned above, synergistic effects of UCN-01 have been observed with many chemotherapeutic agents, including mitomycin C, 5-fluorouracil, carmustine, and camptothecin [3, 16, 63, 64, 67, 103, 133, 142, 151]. Therefore it is possible that combining UCN-01 with these or other agents could improve its therapeutic index. Clinical trials exploring these possibilities are being developed.

UCN-01 administered by an intravenous or intraperitoneal route displayed antitumor activity in xenograft model systems with breast carcinoma (MCF-7 cells), renal carcinoma (A498 cells), and leukemia (MOLT-4 and HL-60) cells (A.M. Senderowicz, unpublished data). The antitumor effect was greater when UCN-01 was given over a longer period. This requirement for a longer period of treatment was also observed in in vitro models, with greatest antitumor activity observed when UCN-01 was present for 72 h [131]. Thus a clinical trial using a 72-h continuous infusion every 2 weeks was conducted.

Clinical trials of UCN-01

The first phase I trial of UCN-01 has been completed [111, 128]. UCN-01 was initially administered as a 72-h continuous infusion every 2 weeks based on data from in vitro and xenograft preclinical models. However, it became apparent in the first few patients that the drug had an unexpectedly long half-life (about 30 days). This half-life was 100 times longer than the half-life observed in preclinical models, most probably due to the avid binding of UCN-01 to α_1-acid glycoprotein [51, 110]. Thus the protocol was modified to administer UCN-01 every 4 weeks (one half-life) and for subsequent courses the duration of infusion was decreased by half (total 36 h). Thus it was possible to reach similar peak plasma concentrations in subsequent courses with no evidence of drug accumulation. There was no evidence of myelotoxicity or gastrointestinal toxicity (prominent adverse effects observed in animal models), despite the high plasma concentrations achieved (35–50 μM) [51, 110, 111, 128]. Dose-limiting toxicities were nausea/vomiting (amenable to standard antiemetic treatments), symptomatic hyperglycemia associated with an insulin-resistance state (increase in insulin and C-peptide levels while receiving UCN-01), and pulmonary toxicity characterized by substantial hypoxemia without obvious radiological changes.

The recommended phase II dose of UCN-01 given on a 72-h continuous infusion schedule was 42.5 mg/m^2 per day [111]. One patient with refractory metastatic melanoma developed a partial response that lasted 8 months. Another patient with refractory anaplastic large-cell lymphoma that had failed multiple chemotherapeutic regimens including high-dose chemotherapy had no evidence of disease 4 years after the initiation of UCN-01. Moreover, a few patients with leiomyosarcoma, non-Hodgkin's lymphoma, and lung cancer demonstrated stable disease for ≥6 months [111, 129]. One patient with refractory large-cell lymphoma that failed prior high-dose combination chemotherapy protocol EPOCH-2 (high-dose infusional CHOP followed by VP-16)

combination chemotherapy had rapidly progressive disease after one cycle of UCN-01. He required immediate systemic salvage chemotherapy due to hepatic and bone marrow failure (thrombocytopenia) caused by progression of disease. Based on the poor status of this patient, a dose-reduced EPOCH combination chemotherapy was administered. His liver function and thrombocytopenia resolved completely with significant improvement in performance status within 2 weeks after combination chemotherapy. Unfortunately, he developed *Candida krusei* septicemia and died. His postmortem examination revealed a pathological complete response after only one cycle of chemotherapy [163]. Thus this patient with refractory large-cell lymphoma became "chemotherapy-sensitive" after only one dose of UCN-01. This phenomenon recapitulates the synergistic effect observed in preclinical models with several chemotherapeutic agents. Several combination trials are being developed based on this observation.

To estimate "free UCN-01 concentrations" in body fluids, several approaches were considered. Plasma ultracentrifugation and salivary determination of UCN-01 revealed similar results. At the recommended phase II dose (37.5 mg/m^2 per day over 72 h), concentrations of "free salivary" UCN-01 (about 100 n*M*) that may cause G_2 checkpoint abrogation can be achieved. As mentioned above, UCN-01 is a potent PKC inhibitor. To determine the putative signaling effects of UCN-01 in tissues, bone marrow aspirates and tumor cells were obtained from patients before and during the first cycle of UCN-01 administration. Western blot studies were performed in those samples against phosphorylated adducin, a cytoskeletal membrane protein, a specific substrate phosphorylated by PKC [49]. Clear loss in phospho-adducin content in the posttreatment samples was observed in all tumor and bone marrow samples tested, and it was concluded that UCN-01 can modulate PKC activity in tissues from patients in this trial [111, 129].

Several groups are conducting shorter duration (3-h) infusional trials of UCN-01. Interestingly, the toxicity profile of shorter infusions is similar to the toxicities observed with the 72-h infusion trial [37, 146]. However, with shorter infusions, more pronounced hypotension was observed [37, 146]. Determination of free UCN-01 in these trials is of utmost importance as higher free concentrations for shorter periods may be more or less beneficial compared with the free concentrations observed in the 72-h infusion trial.

Based on the unique pharmacological features and anecdotal clinical evidence of synergistic effects in one patient with refractory disease [163], several combination trials with standard chemotherapeutic agents have commenced. A phase I/II trial of gemcitabine followed by 72-h infusional UCN-01 in CLL has been initiated at the NCI. Other studies of UCN-01 in combination with cisplatin or 5-fluorouracil, among other agents, have also commenced.

Summary

Based on the frequent aberration in cell-cycle regulatory pathways in human cancer by "CDK hyperactivation," novel ATP competitive CDK inhibitors are being developed. The first two tested in clinical trials, flavopiridol and UCN-01, showed promising results with evidence of antitumor activity and plasma concentrations sufficient to inhibit CDK-related functions. The optimal schedule to be administered, combination with standard chemotherapeutic agents, best tumor types to be targeted, and demonstration of CDK modulation from tumor samples from patients in these trials are important issues that need to be answered to further advance these agents to the clinical arena.

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